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Characterization of a novel protein of *Leptospira interrogans* exhibiting plasminogen, vitronectin and complement binding properties

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ABSTRACT

Leptospirosis is a severe zoonosis caused by pathogenic species of the genus *Leptospira*. This work focuses on a hypothetical protein of unknown function, encoded by the gene LIC13259, and predicted to be a surface protein, widely distributed among pathogenic leptospiral strain. The gene was amplified from *L. interrogans* serovar Copenhageni, strain Fiocruz L1-130, cloned and the protein expressed using *Escherichia coli* as a host system. Immunofluorescence assay showed that the protein is surface-exposed. The recombinant protein LIC13259 (rLIC13259) has the ability to interact with the extracellular matrix (ECM) laminin, in a dose-dependent manner but saturation was not reach. The rLIC13259 protein is a plasminogen (PLG)-binding protein, generating plasmin, in the presence of urokinase PLG-activator uPA. The recombinant protein is able to mediate the binding to human purified terminal complement route vitronectin, C7, C8 and C9, and to recruit and interact with these components from normal human serum (NHS). These interactions are dose-dependent on NHS increased concentration. The binding of rLIC13259 to C8 and vitronectin was slight and pronounced inhibited in the presence of increasing heparin concentration, respectively, suggesting that the interaction with vitronectin occurs via heparin domain. Most interesting, the interaction of rLIC13259 with C9 protein was capable of preventing C9 polymerization, suggesting that the membrane attack complex (MAC) formation was inhibited. Thus, we tentatively assign the coding sequence (CDS) LIC13259, previously annotated as unknown function, as a novel protein that may play an important role in the host's invasion and immune evasion processes, contributing to the establishment of the leptospiral infection.

1. Introduction

Leptospirosis is a zoonosis caused by pathogenic species of the genus *Leptospira*. In urban settings, the most prevalent causal agent of the disease in humans is L. interrogans, having Rattus novergicus as the main host. The bacterial transmission may occur directly by animal contact or indirectly, through contact with soil or contaminated water. Humans are accidental and terminal hosts in the transmission process of leptospirosis (Bharti et al., 2003; Faine et al., 1999).

The genomes of pathogenic *Leptospira* have been sequenced (Ren et al., 2003; Nascimento et al., 2004a,b; Bulach et al., 2006) and *in silico* analysis identified more than 200 predicted outer membrane proteins (Nascimento et al., 2004a,b). Functional genomics studies, including transcription profiles, gene cloning, protein expression and binding characterization to host's components, supplement the *in silico* analysis and help understanding the bacterial pathogenesis (Palaniappan et al., 2007). These proteins are potential targets for inducing immune responses during infection and, therefore, constitute targets for immune protection through mechanisms such as antibody-dependent phagocytosis and killing mediated by complement. In addition, it is possible that a number of these membrane proteins mediates the initial adhesion to host cells (Merien et al., 2000; Barbosa et al., 2006; Choy et al., 2007; Stevenson et al., 2007; Atzingen et al., 2008; Hoke et al., 2008; Hauk et al., 2008).

Indeed, we have reported a number of ECM-binding proteins that potentially contribute for leptospires infection process (Vieira et al., 2014). Following adherence, the bacteria have to overcome the host barriers, to reach the bloodstream and secondary sites of infection.

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Proteolytic activities by diverse mechanisms, including plasmin generation on bacterial surface and matrix metalloproteases (MMPs) activation seems to help *Leptospira* trespassing monolayer cells by ECM, IgG and C3b degradation (Vieira et al., 2009, 2011; Vieira et al., 2013). It has been shown that virulent *Leptospira,* but not the saprophyte, can evade the host's innate immune system by binding complement regulators, such as vitronectin, factor H (FH), factor H-like 1 (FHL-1), factor H-related 1 (FHR-1), C4b binding protein (C4BP) (da Silva et al., 2015; Meri et al., 2005; Castiblanco-Valencia et al., 2012; Siqueira et al., 2016, 2017), suggesting possible mechanisms that could help virulent *Leptospira* evade the host's immune system.

In the present work, we investigated the ability of a novel leptospiral protein encoded by the gene LIC13259 to bind human host's components. We show that the coding sequence LIC13259 is possibly located at the bacterial surface and the expressed recombinant protein has the ability to interact with human laminin, plasminogen and components of terminal complement system pathway. The characterization of this multifaceted protein and the implication of these interactions for the leptospiral host-infection are here addressed.

2. Material and methods

2.1. Biological components

Collagen, laminin, plasma and cellular fibronectin, elastin, fibrinogen, human vitronectin and the control proteins BSA, gelatin and fetuin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Laminin-1 and collagen type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma; cellular fibronectin was derived from human foreskin fibroblasts; elastin was derived from human aorta, and collagen type I was isolated from rat-tail. Native plasminogen, purified from human plasma, and factor H were purchased from EMD chemicals (San Diego, CA, USA). C4BP, C6, C7, C8 e C9 isolated from normal human serum, were purchased from Complement Technology (Tyler, TX, USA).

2.2. Bacterial strains and serum samples

The non-pathogenic *L. biflexa* (serovar Patoc strain Patoc 1), the pathogenic and virulent *L. interrogans* (serovar Copenhageni strain L1-130), the pathogenic attenuated *L. interrogans* (serovars Canicola strain Hond Utrecht IV, Copenhageni strain M20 were cultured 28 °C under aerobic conditions in liquid EMJH medium containing asparagine (0.015% w/v), sodium pyruvate (0.001% w/v) calcium chloride (0.001% w/v), magnesium chloride (0.001% w/v), peptone (0.03% w/v) and meat extract (0.02% w/v) (Turner, 1970). *Leptospira* cultures are maintained in the Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. *E. coli* DH5α and *E. coli* BL21(DE3) (Mendes et al., 2011) were used as cloning and recombinant protein expression hosts, respectively. Leptospiral DNA extraction was performed as previously described (Oliveira et al., 2011).

2.3. In silico analysis of the coding sequences LIC13259

The LIC13259 coding sequence (CDS) was selected from *L. interrogans* serovar Copenhageni genome sequences (Nascimento et al., 2004a, b) based on their cellular localization prediction by CELLO, http://cello.life.nctu.edu.tw/ (Yu et al., 2006, 2010). The signal peptide sequence was assessed by SignalP, http://cbs.dtu.dk/services/SignalP-3.0/ (Bendtsen et al., 2004), and LipoP, http://www.cbs.dtu.dk/services/LipoP/ (Juncker et al., 2003). The Smart, http://smart.embl-heidelberg.de/ (Letunic et al., 2015; Schultz et al., 1998), and PFAM, http://pfam.xfam.org/ (Finn et al., 2006), web servers were used to search for predicted functional and structural domains within the amino acid sequence. Multiple sequence alignment was performed with ClustalW2 (Larkin et al., 2007), comparing LIC13259 with the sequences available in GenBank.

Table 1
Oligonucleotides used for qPCR study.

Gene	Primer sequence	Tm (ºC)
LIC13259	F: 5' CGCTCATGTAGTAGAGCTCGTT 3'	58
	R: 5' TGAAAACTCCTGTTCCCCCT 3'	58
16S	F: 5' CACGAAAGCGTGGGTAGTGA 3'	58
	R: 5' CAACGTTTAGGGCGTGGATTA 3'	58

2.4. RNA extraction and real-time reverse transcriptase quantitative PCR (RT-qPCR)

Leptospiral cells were recovered from liquid EMJH culture medium by centrifugation (3075 × *g*, 15 min, 4 °C) and total RNA was extracted using Trizol reagent (Invitrogen), as recommended by the manufacturer. Residual DNA was eliminated by incubation with DNAseI (Invitrogen) and the cDNAs were obtained after reverse transcriptase PCR amplification of RNAs using SuperScript III kit Reverse Transcriptase (Invitrogen). RT-qPCR was performed using CFX96 Real-Time System (Bio-Rad) and SYBR Green I dye (Applied Biosystems) to detect synthetized double strand DNAs, employing primer pair described in Table 1. Reactions were performed with SYBR Green PCR Master Mix (Applied Biosystems) in a 20 µL reaction volume. Cycle parameters: 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 58 °C for 1 min. The relative gene expression among leptospiral strains was performed using comparative $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

2.5. Cloning, expression and purification of recombinant proteins in E. coli

The gene LIC13259 without the signal peptide was amplified from the genomic DNA of *L. interrogans* serovar Copenhageni by PCR with specific primers. The PCR amplified product was cloned into pGEM-T easy vector (Promega) and positive clones were selected by DNA restriction analysis, PCR of colonies and further confirmed by nucleotide sequencing analysis. After confirmation of the sequences, the DNA inserts were removed by digestion with the specific restriction enzyme and ligated into the protein expression pAE vector (Ramos et al., 2004), previously digested with the same enzymes. This plasmid includes a 6XHis sequence tag at the N-terminus of recombinant proteins. The plasmid pAE-LIC13259 confirmed by sequencing, was employed to transform BL21 (DE3) expression host cells. *E. coli* BL21 (DE3) cells containing the construction were grown at 37 °C in Luria-Bertani (LB) broth with 100 µg/ml ampicillin.

2.6. Circular dichroism (CD) spectroscopy

Measurements were obtained by circular dichroism (CD) spectroscopy at room temperature in a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). CD spectroscopy of the far-UV spectrum for recombinant protein was performed in 10 mM sodium phosphate buffer. The spectra were measured and are presented as the average of five scans recorded from 190 to 260 nm and the residual molar ellipticity was expressed in degree cm^2 $dmol^{-1}$. Spectral data were analyzed with the software CAPITO for estimation of the secondary structure content (http://capito.nmr.leibniz-fli.de/) (Wiedemann et al., 2013).

2.7. Prediction of three-dimensional structure of the protein

The three-dimensional structure of the LIC13259 protein was modeled using the I-TASSER server (Wu et al., 2007). The sequence corresponding to the peptide signal was removed. The I-TASSER server uses many crystal structures to generate five models. The best model was selected from the C-Score. The figure was generated using the

Pymol program.

2.8. Antiserum production in mice against recombinant protein

Female BALB/c mice (4–6 weeks old) were immunized subcutaneously with ten micrograms of recombinant protein adsorbed in 10% (vol/vol) of Alhydrogel [2% Al(OH)3; Brenntag Biosector], used as adjuvant. Two subsequent booster injections were given at 2-week intervals with the same preparation. Negative control mice were injected with PBS/adjuvant. Two weeks after each immunization, the mice were bled from retro-orbital plexus and pooled sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers. Prior to experiments, anti-recombinant protein sera were adsorbed with a suspension of *E. coli* to suppress the reactivity of anti-*E. coli* antibodies (Gruber and Zingales, 1995).

2.9. Immunoblotting analysis

The purified rLIC13259 protein was loaded into 12% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare). The membranes were blocked overnight with 10% skimmed dry milk in PBS containing 0.05% Tween 20 (PBS-T), washed 3 times and then incubated with anti-rLIC13259 (1:5000) mouse polyclonal serum for 1 h at RT. Next, before washes, the membranes were incubated with HRP-conjugated anti-mouse IgG (1:5000 Sigma) for 1 h at room temperature. Monoclonal HRP-conjugated anti-his tag antibodies (1:10,000) were also used. The protein reactivity was revealed by ECL reagent kit (GE Healthcare) as previously described (Fernandes et al., 2015).

2.10. Immunofluorescence assay (IFA)

The localization of LIC13259 CDS by IFA was performed as follows: *L. interrogans* serovar Copenhageni suspensions containing approximately 10^9 cells/mL of live leptospires were harvested at 3800 × *g* for 15 min, washed twice with PBS (with 50 mM NaCl), resuspended in 200 µl of PBS with 2% paraformaldehyde for 40 min at 30 °C under gentle shaking. After incubation, the leptospires were washed with PBS and incubated for 2 h at 30 °C with polyclonal mouse anti-serum at 1:50 dilution against rLIC13259 and LipL46, used as positive control (Santos et al., 2018). FliG, a cytoplasmic flagellar protein (Levenson et al., 2012) and pre-immune serum were employed as negative controls. The leptospires were washed (PBS containing 5% BSA) and incubated with anti-mouse IgG antibodies conjugated to fluorescein isothiocyante (FITC, Sigma) at 1:50 dilution and propidium iodide diluted 40X (Sigma- Aldrich) for 16 h (Santos et al., 2018; Oliveira et al., 2008). Leptospires were then washed and resuspended in 50 µl of PBS plus 50 µl of anti-fading solution (ProLong Gold, Molecular Probes) for total volume of 100 µl. The immunofluorescence-labeled leptospires were examined using a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany).

2.11. Detection of native protein in leptospiral surface

Suspensions of 10 mL live leptospires (˜10^8 cells/ml per treatment) were harvested at 3800 g for 15 min at room temperature and washed twice with PBS (with 50 mM NaCl). Leptospires were incubated for 2 h at 30 °C with polyclonal mouse anti-serum against rLIC13259 (1:200), DnaK (Haake and Matsunaga, 2002) and PBS at 1:100 dilution. The leptospires were washed (PBS containing 1% BSA) and incubated with anti-mouse IgG antibodies conjugated to fluorescein isothiocyante (FITC, Sigma) at 1:100 dilution for 2 h. After this period, the leptospires were subsequently centrifuged at 3800 g for 15 min, washed twice with PBS, resuspended in PBS and distributed onto black plate. The immunofluorescence intensity was examined using a Fluoroskan Ascent FL (Thermo Scientific).

2.12. Binding of recombinant protein to ECM, plasma and complement components

Attachments of recombinant protein to individual ECM, plasma and complement components were analyzed according to a previously published protocol (Atzingen et al., 2008), with some modifications. Briefly, 96-well plates were coated with 1 µg laminin, collagen type I, collagen type IV, cellular and plasma fibronectin, elastin, human PLG, fibrinogen, C6, C7, C8, C9, vitronectin, factor H, C4BP in 100 µl PBS for 16 h at 4 °C. Fetuin and BSA were employed as negative controls. The wells were washed three times with PBS-T and then blocked with 200 µl PBS-T containing 1% BSA for 2 h at 37 °C. One microgram of each recombinant protein in 100 µl PBS-T containing BSA (1%) was added to each well, and protein was allowed to attach to different components for 2 h at 37 °C. After washing with PBS-T, bound recombinant proteins were detected by adding 1:5000 dilution of mouse serum against protein in 100 µl PBS-T/BSA (1%). Incubation proceeded for 1 h at 37 °C. After washings with PBS-T, 100 µl 1:10,000-diluted HRP-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) in PBS-T/BSA (1%) was added per well followed by incubation for 1 h at 37 °C. In addition, antiserum against each component was employed to probe protein binding. The wells were washed three times and OPD (Sigma-Aldrich) (1 mg/ml) in citrate phosphate buffer (pH 5.0) plus 1 µl/mL H_2O_2 was added (100 µl per well). The reaction was allowed to proceed for 15 min and then interrupted by the addition of 50 µl 2 M H_2SO_4. Readings were taken at 492 nm in a microplate reader (Multiskan EX; Thermo Fisher).

2.13. Dose-response curves

First, 96-wells plates were coated with 1 µg laminin, PLG, C7, C8, C9 and vitronectin (1 µg each) in 100 µl PBS overnight at 4 °C. Plates were then blocked and increasing concentrations of the purified rLIC13259 were added (100 µl per well, in PBS-T containing 1% BSA). The assessment of bound protein was performed by incubation for 1 h at 37 °C with the anti-rLIC13259 (1:5000 dilution in PBS-T containing 1% BSA). After washings with PBS-T, 100 µl 1:10,000-diluted HRP-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) in PBS-T/BSA (1%) was added per well followed by incubation for 1 h at 37 °C. The reaction was revealed with OPD substrate as described above.

2.14. Characterization of protein binding to PLG and plasmin enzymatic activity assay

Evaluation of the role of lysine residues in the binding of recombinant protein to PLG, and plasmin generation were performed as described in (Vieira et al., 2009).

2.15. Characterization of protein binding to complement components

For investigation of the interference of heparin on interaction of rLIC13259 with complement components, microtiter plates were coated with rLIC13259 (10 µg/mL) for 16 h at 4 °C. The wells were washed with PBS, blocked with PBS-1% BSA for 2 h at 37 °C, and incubated with purified C7, C8, C9 or vitronectin (10 µg/mL) in the presence of increasing amounts of heparin (0–500 µg/mL). Reaction mixtures were incubated for 60 min at 37 °C. Unbound components was removed by three washes with PBS-T, and bound was detected by the use of goat anti-human C7, C8 and C9 (1:10,000) or rabbit anti-human vitronectin (1:5000), respectively, followed by peroxidase-conjugated anti-goat IgG (1:10,000) or anti-rabbit IgG (1:5000). Detection was performed with OPD (Sigma-Aldrich), and absorbance was measured at 492 nm.

2.16. Interaction of denatured recombinant rLIC13259 with PLG

Firstly, ELISA plates were coated with components (10 µg/mL) for

16 h at 4 °C. Plates were washed three times with PBS-T and blocked with PBS-1% BSA for 2 h at 37 °C. Two and a half micrograms of recombinant protein rLIC13259 was denatured by incubation at 96 °C for 10 min before being added, in 100 µl PBS, to each well. The rLIC13259 was allowed to attach to PLG at 37 °C for 2 h. After washing with PBS-T, bound recombinant protein was detected by incubation with mouse anti-rLIC13259 (1 : 5000) at 37 °C for 1 h, followed by HRP-conjugated rabbit anti-mouse IgG (1:5000) (Sigma). Detection was performed with OPD (Sigma-Aldrich), and absorbance was measured at 492 nm.

2.17. Assessment of PLG, C7, C8, C9 and vitronectin recruitment by rLIC13259 from NHS

Protein attachments to serum PLG, C7, C8, C9 and vitronectin were analyzed by ELISA. Microtiter plates were coated overnight at 4 °C with 1 µg of recombinant protein or gelatin (negative control). The wells were washed with PBS containing 0.05% Tween 20 (PBS-T), blocked with 300 µl of buffer solution Super Block T20 (TBS) (Thermo Fisher Scientific) for 40 min at 25 °C and incubated with normal human serum (NHS) diluted in the blocking solution (0–30%) for 90 min at 25 °C. After washing, components binding to recombinant proteins were detected by adding mouse serum against each 1:5000 diluted component in blocking solution for 1 h at 37 °C. After washing the plates, 100 µl of a solution containing horseradish peroxidase (HRP)-conjugated anti-IgG corresponding of each component were added to each well and incubation proceeded for 1 h at 37 °C. The wells were washed and reactivity revealed by using OPD substrate as above mentioned.

2.18. C9 polymerization assay

The effect of rLIC13259 on C9 polymerization was assessed according to a previously published protocol (Zhang et al., 2011). Briefly, rLIC13259 (1.25–5 µg) and the negative-control protein BSA (2.5 µg) were pre-incubated with 3 µg of C9 at 37 °C in 20 mM Tris-HCl (pH 7.4). After 40 min incubation, 50 M $ZnCl_2$ in 20 mM Tris-HCl (pH 7.2) was added for 2 h at 37 °C. The samples were separated in a precast 4–20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA), and C9 polymerization was visualized by comassie blue staining.

2.19. Statistical analysis

All results are expressed as the mean ± SD. Student's paired *t*-test was used to determine the significance of differences between means and $P < 0,05$ was considered statistically significant.

2.20. Ethics statement

All animal studies were approved by the Ethical Committee for Animal Research of the Instituto Butantan, Brazil, under protocol no. 4330120116. The Committee for Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation (COBEA).

3. Results

3.1. In silico analysis of LIC13259 coding sequence, conservation among leptospiral strains and gene expression

The CDS LIC13259 was selected from the genome of *L. interrogans* serovar Copenhageni based on *in silico* analysis. The protein coded by the gene LIC13259 has been annotated as a probable lipoprotein, comprising an N-terminus sequence recognized by the SpII (signal peptidase) machinery, responsible for the covalent attachment of a fatty acid at the cysteine N-terminus of the proteins (Juncker et al., 2003). The predicted cleavage site for this protein is between amino acids 26-27. CELLO software predicts the location of LIC13259 protein at

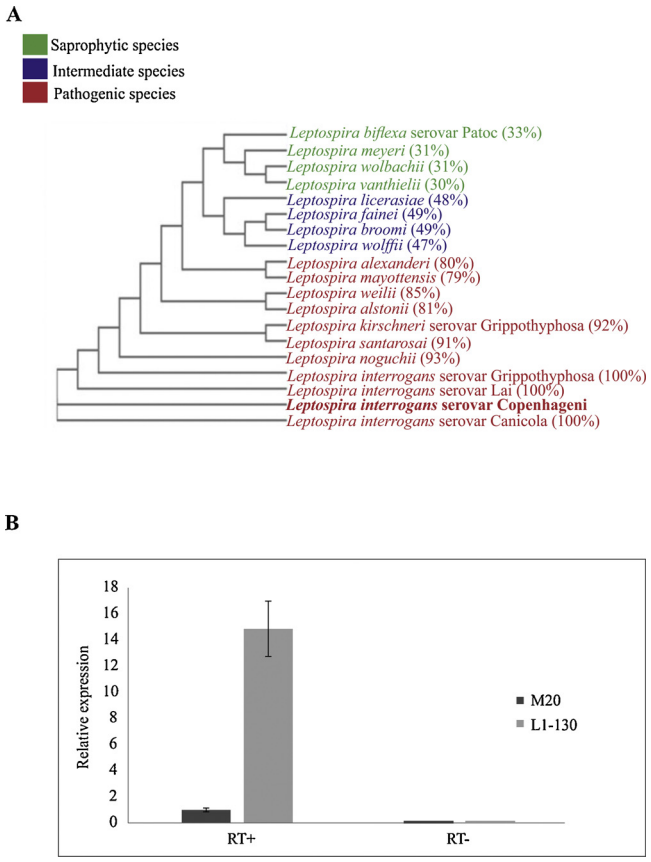

Fig. 1. Analysis of LIC13259 coding sequence conservation among *Leptospira* strains *in silico* and RT-qPCR. Sequence conservation among Leptospira spp. BLAST analysis was performed among sequences of amino acids available in GenBank database and leptospiral sequences were employed to perform Clustal Omega multiple sequences alignments. The resulting phylogram shows the high level of sequence conservation (A). LIC13259 gene expression was analyzed in the culture-attenuated (*L. interrogans* serovar Copenhageni, strain M20) and virulent (*L. interrogans* serovar Copenhageni, strain Fiocruz L1-130) by qPCR. The RT- was used as negative control (B).

periplasmic space of the bacteria (Yu et al., 2006). Domain of unknown function (DUF3347) was found within the protein according to PFAM and SMART webservers (Finn et al., 2006; Schultz et al., 1998). ClustalW2 multiple sequence alignment (Fig. 1A) shows a high level of sequence conservation among pathogenic strains of *Leptospira*, contrasting with the low similarity with the sequences present in saprophyte strains, about 30%.

Gene expression levels in virulent *L. interrogans* strain L1-130 and culture-attenuated *L. interrogans* strain M20 (Fig. 1B) was compared by RT-qPCR. The results revealed the presence of LIC13259 mRNAs in both strains, with a higher protein expression in virulent L1-130 compared to the culture-attenuated strain, of approximately 10 fold-changes. Furthermore, quantitative proteomics analysis of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, detected this protein but the number of copies per cell was below the detection limit (Malmström et al., 2009).

3.2. Protein expression, purification and structural integrity analyses

Oligonucleotides were designed based on genome sequences of *L. interrogans* serovar Copenhageni for amplification of the coding sequence LIC13259. Amplified DNA fragment was cloned into the pAE vector and sequence analysis verified that the insert was cloned in frame. Induction of recombinant protein expression was performed in *E. coli* BL21 DE3 strain with IPTG. The resulting recombinant protein was

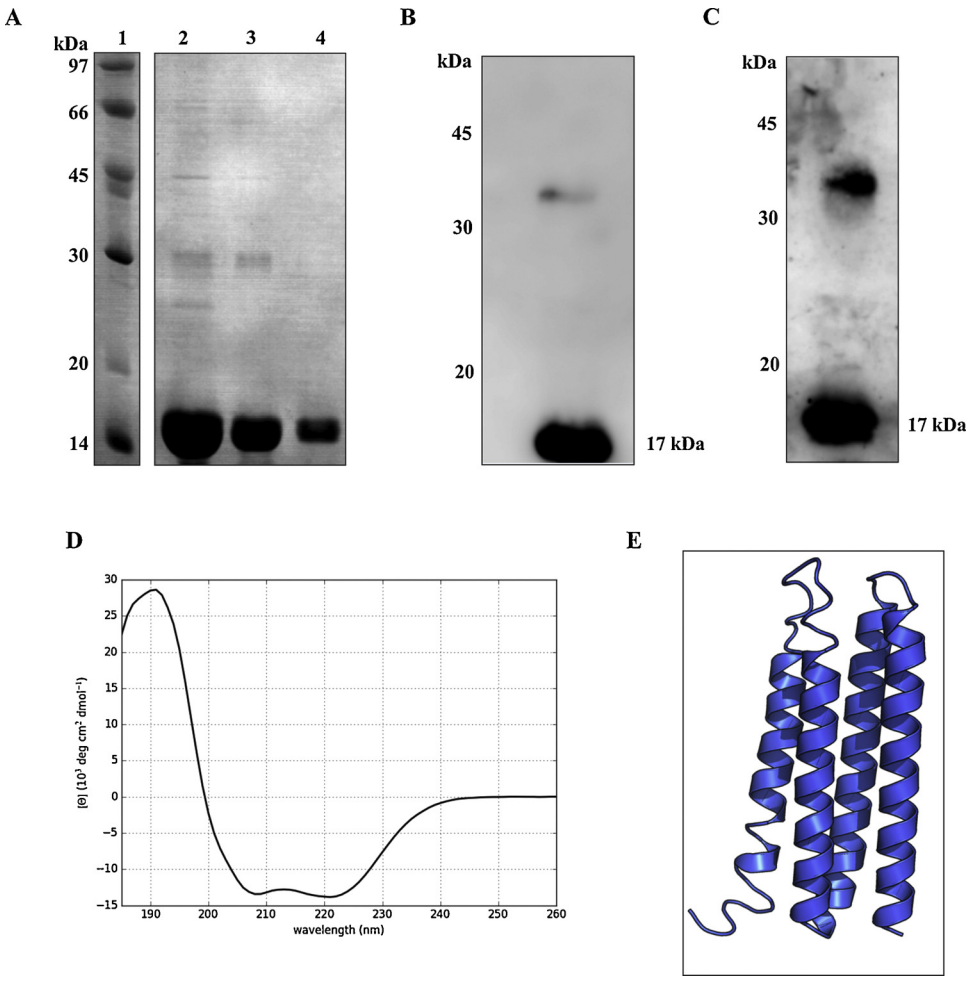

Fig. 2. Analysis of recombinant protein by SDS-PAGE, Western blotting and CD spectroscopy. (a) Purified recombinant protein. 1, molecular protein mass marker (kDa); 2, 3 and 4, purified recombinant protein fraction after dialysis. (b) Western blotting analyses of recombinant protein using the anti-His MAbs (1:10,000). (c) Western blotting analyses of recombinant protein using the corresponding antiserum produced in mice (1:5000). (d) CD spectra of rLIC13259 recombinant protein. Far-ultraviolet CD spectra are shown as an average of five scans from 190 to 260 nm. (e) Figure generated using the Pymol program from the molecular modeling performed by the I-TASSER server.

expressed in its soluble form with 6X His tag at the N-terminal. LIC13259 recombinant protein (rLIC13259) was purified by immobilized metal ion affinity chromatography and recovered with 0.5 M imidazol. The recombinant protein band was analyzed by SDS-PAGE and showed a 17 kDa estimated molecular mass (Fig. 2A). rLIC13259 protein was confirmed by probing Western blot with anti-His MAbs (Fig. 2B) and homolog polyclonal antibodies raised in mouse. Western blotting analysis suggests that rLIC13259 seems to form a dimer, with a recognized molecular mass above 30 kDa (Fig. 2C). Structural integrity of purified protein was assessed by circular dichroism spectroscopy (Fig. 2D). The CD spectrum shows negative bands at 222 nm and 208 nm and a positive band at approximately 193 nm, which is consistent with α- helical secondary structure content. Analysis of the spectrum data by CAPITO software corroborates with the experimental data showing an alpha helix contents of 99%. Three-dimensional structure prediction of LIC13259 protein was modeled using the I-TASSER server. The figure was generated using the Pymol program (Fig. 2E) showed the predominance of the α- helical secondary structure.

3.3. Cellular localization of the native protein on the L. interrogans

The CDS LIC13259 is predicted to be a membrane protein. Thus, to evaluate if rLIC13259 is located at the bacterial surface immunofluorescence confocal microscopy was performed. Leptospires were fixed and treated with the corresponding polyclonal antiserum, raised in mice against protein, followed of anti-mouse IgG antibodies conjugated to FITC. Polyclonal antibody against LipL46, a leptospiral surface antigen, was used as positive control while antibodies against FliG, a cytoplasmic flagellar protein, and pre-immune serum, were used as negative controls. Green fluorescence distributed along the bacteria could be observed for rLIC13259 and LipL46 (Fig. 3A – second column). Leptospires were visualized by propidium iodide staining (Fig. 3A – first column). The localization of the protein at cell surface was achieved by merging both fields (Fig. 3A – third column).

In another assay, intact leptospires were incubated with polyclonal antiserum against rLIC13259 protein to evaluate whether these antibodies might recognize native protein at surface of intact bacteria. DnaK, a cytoplasmic protein and pre-immune serum, was used as negative control. Furthermore, controls lacking leptospires or the primary antibody were used. After treatment of leptospires with corresponding polyclonal antiserum, anti-mouse IgG antibodies conjugated to FITC were used and fluorescence intensity was measured. The presence of rLIC13259 orthologs in *L. biflexa* saprophyte strain was also evaluated. As showed in Fig. 3B, higher fluorescence intensity was observed for rLIC13259 when compared to the treatment performed with DnaK or with pre-immune antiserum. Basal level of fluorescence was observed for groups treated with saprophyte strain, suggesting the recognition of the native protein only at surface of pathogenic leptospires.

3.4. Characterization of rLIC13259 binding to ECM components

The rLIC13259 protein is suggested by bioinformatics and immunofluorescence microscopy to be surface-exposed. Thus, the ability of this protein to mediate host colonization by adhering to extracellular matrix proteins was examined by ELISA. Laminin, cellular fibronectin, collagen I, collagen IV and elastin were immobilized on microdilution plates and rLIC13259 attachment was evaluated using antiserum

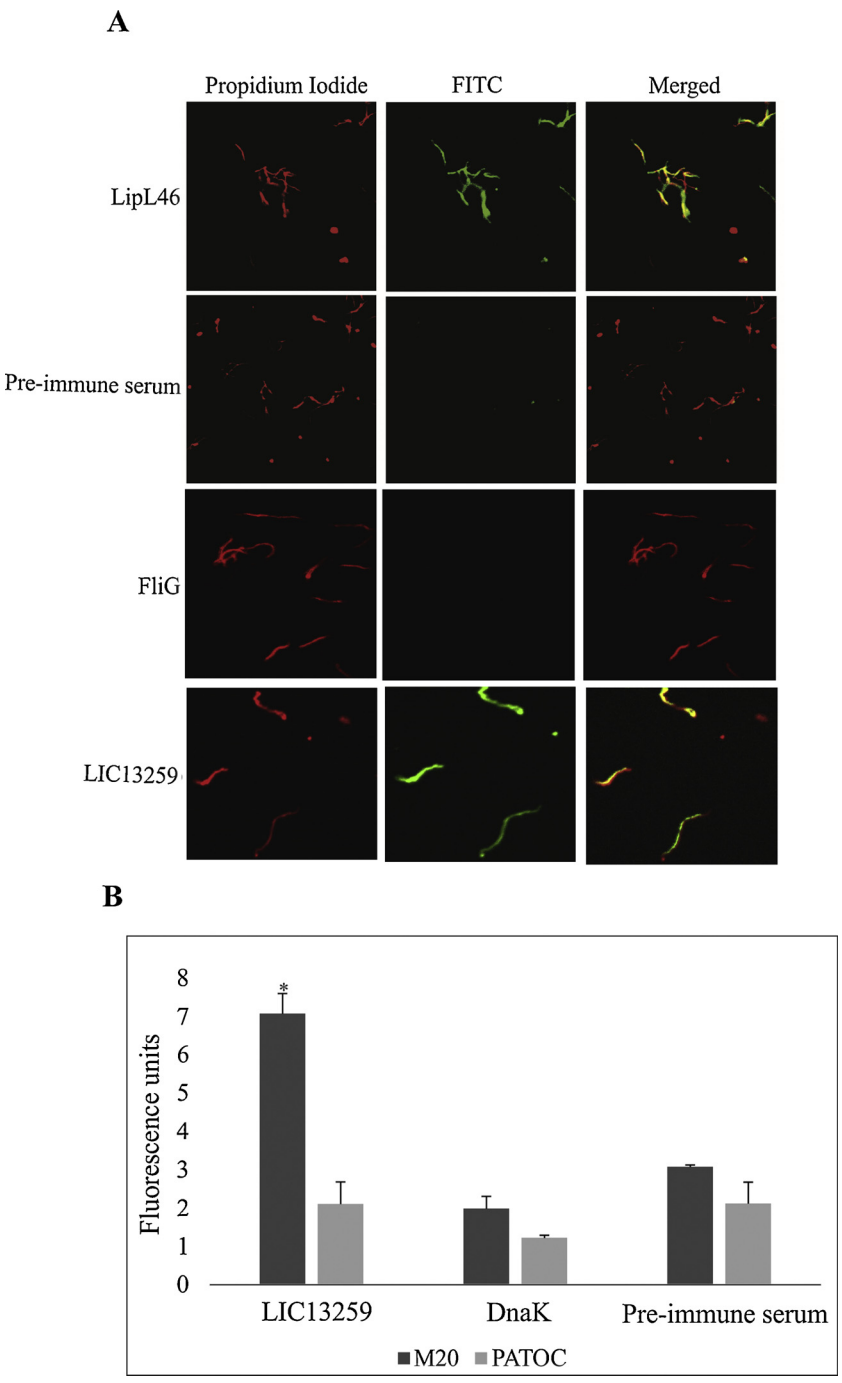

Fig. 3. Cellular localization of LIC13259 CDS in *L. interrogans* by fluorescence assays. (a) *L. interrogans* serovar Copenhageni strain M20 was fixed by paraformaldehyde and incubated with antisera anti-rLIC13259, anti-LipL46 (positive control, exposed protein) or anti-FliG and pre-immune serum (negative controls). Propidium iodide (PI) and FITC-conjugated secondary antibodies were added to identify leptospiral DNA and surface-bound antibodies, respectively. Cellular location is shown in the merged fields. (b) Leptospires were incubated for 2 h at 30 °C with polyclonal mouse anti-serum against rLIC13259 (1:200), DnaK (negative control, non-exposed protein) and pre-immune serum (negative control), followed by FITC-conjugated secondary antibody. The immunofluoresce intensity was examined using a Fluoroskan. For statistical analysis, the groups were compared using test-t Student, comparing data of DnaK with each coding sequence, (*$p < 0.05$).

against the recombinant protein. BSA and fetuin were used as negative control. The rLIC13259 protein exhibited adhesiveness only to laminin when compared to binding with the negative controls, BSA and fetuin. No binding was observed when wells were coated with cell fibronectin, collagen and elastin (results not shown). Although a dose-dependent binding was observed when rLIC13259 protein concentration was allowed to react to a fix immobilized laminin concentration, saturation level was not reached even using a high protein concentration (results not shown), suggesting that this interaction is probably not relevant.

3.5. Interaction of rLIC13259 to human plasma components

Bacterial pathogens have evolved to a wide range of strategies to colonize and invade human organs. It has been shown that *Leptospira* bind PLG and that several proteins at the bacterial surface could act as PLG receptors (Vieira et al., 2009, 2010a; Fernandes et al., 2015). Moreover, it has been demonstrated that leptospires are able to interact with human fibrinogen, reducing the formation of fibrin clot in a thrombin-catalyzed reaction (Oliveira et al., 2013). Proteins binding to plasma fibronectin have also been shown (Atzingen et al., 2008; Pinne et al., 2010; Teixeira et al., 2015). Thus, to evaluate if the rLIC13259 is capable of binding human PLG, fibrinogen, plasma fibronectin, BSA and fetuin, these components were immobilized onto microdilution plates followed by incubation with the recombinant protein rLIC13259 and the binding was evaluated using antiserum against the recombinant protein. The results showed that rLIC13259 protein bound only to human PLG (Fig. 4A), and that this interaction was further confirmed using an anti-plasminogen antibody (Fig. 4B). The rLIC13259 interaction with PLG was also confirmed by Western blotting probed with polyclonal antiserum raised against rLIC13259 (Fig. 4C). To

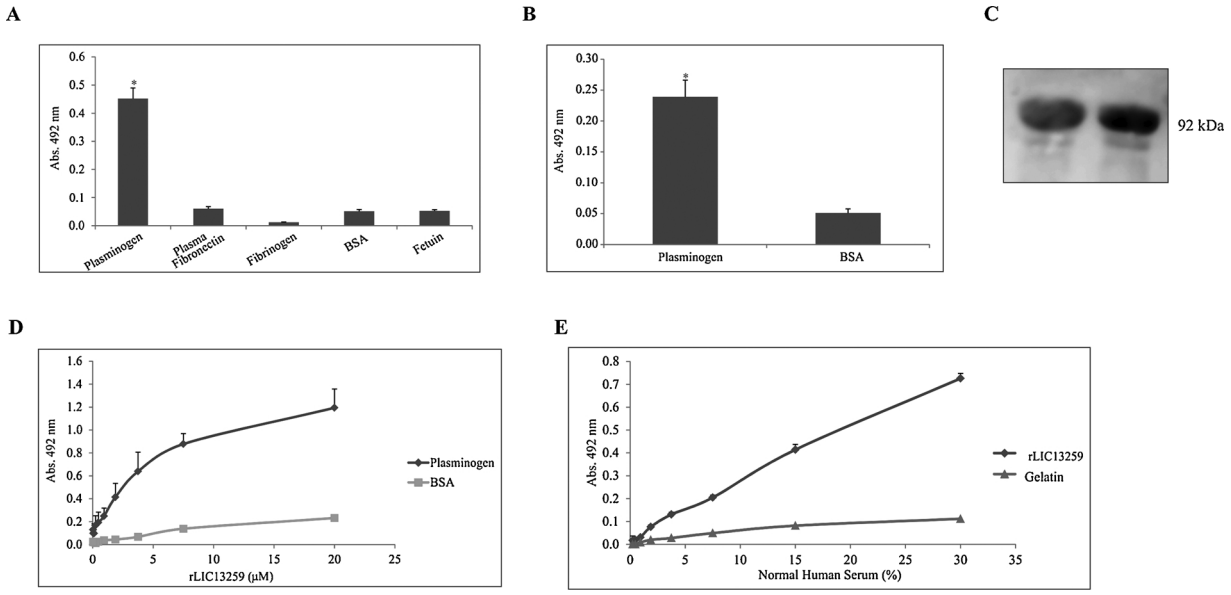

Fig. 4. Binding of recombinant protein with plasma components. (a) ELISA plates were coated with human plasma components or the control proteins fetuin and BSA. Recombinant protein rLIC13259 was added and component binding was detected by incubation with anti-recombinant polyclonal antibodies. (b) ELISA plates were coated with rLIC13259 or the control protein BSA. Plasminogen was added and component binding was detected by incubation with anti-plasminogen polyclonal antibodies. Bars represent the mean ± SD absorbance at 492 nm of three replicates for protein and are representative of two independent experiments. For statistical analyses, the interaction of recombinant protein with plasma components were compared to its binding to BSA (negative control) by two-tailed *t*-test (* $p < 0.05$). (c) Plasminogen and BSA (negative control) were separated by SDS-PAGE, transferred into nitrocellulose membrane, followed by incubation with rLIC13259, and component binding was detected by incubation with anti-recombinant polyclonal antibodies. Both bands correspond to PLG-protein binding. No protein band was detected in the negative control. (d) Dose-dependent binding experiment of recombinant protein to plasminogen was performed by incubation with different protein concentrations (0–20 μM). (e) One microgram of recombinant protein was coated onto microtiter plates and incubated with different amounts of NHS (0–30%). Gelatin was used as negative control for non-specific binding. Binding was detected with antiserum against PLG. Each point represents the mean absorbance at 492 nm ± SD of three replicates and is representative of two independent experiments.

characterize the interaction of rLIC13259 with PLG, increasing concentrations of rLIC13259 were allowed to react with a fixed concentration of PLG. Dose-dependent binding was observed when recombinant protein concentration ranging from 0 to 20 μM was used (Fig. 4D). Despite the high protein concentrations used, saturation was not reached. To assess whether the rLIC13259 protein could recruit PLG from NHS, recombinant protein was immobilized onto microdilution plates and incubated with different dilution of NHS from 0 to 30%. Gelatin was used as negative control. As shown in Fig. 4E, rLIC13259 can acquire PLG from NHS, in a dose-dependent manner, suggesting that binding to PLG by rLIC13259 can potentially occur i*n vivo*.

3.6. Characterization of rLIC13259 binding to human PLG

To assess the involvement of protein structure in the interaction between rLIC13259 and PLG, heat-denatured recombinant protein was used to evaluate the binding. Loss of protein structure reduced almost 50% of the interaction with PLG, suggesting the involvement of structural epitopes on the binding reaction (Fig. 5A). It has been shown that PLG kringle domains participate in the binding of PLG and *L. interrogans* (Vieira et al., 2009) and several PLG- binding proteins mediate these interactions (Teixeira et al., 2015; Pereira et al., 2017; Santos et al., 2018). To verify the participation of lysine residues of rLIC13259 protein on the binding to PLG, increasing concentrations of the lysine-analog ACA were added to the reaction. As depicted in Fig. 5B, 2 mM ACA almost totally abolished the binding of rLIC13259 to PLG, suggesting the participation of these domains in the interaction. It was reported that enzymatically active PLA is generated by PLG bound to leptospiral proteins when its activator is present (Vieira et al., 2009). To assess whether PLG attached to rLIC13259 protein can also achieve proteolytic activity, rLIC13259 was immobilized on microdilution plates and incubated with PLG. The uPA-type PLG activator was added together with a plasmin-specific chromogenic substrate. The plasmin activity was indirectly evaluated by measuring the cleavage of the plasmin-specific chromogenic substrate at 405 nm. As illustrated in Fig. 5C, only the complete system, rLIC13259, PLG, uPA and plasmin substrate, shows the expected plasmin-derived product. No cleavage of the chromogenic substrate was observed in controls lacking at least one of the components of the reaction.

3.7. Interaction of rLIC13259 with human components of the complement system

It is well known that pathogenic bacteria can escape the host's innate immune system, being able to survive and establish an infection. The ability of leptospires to interact with complement regulators and proteins of the terminal complement pathway have been shown and suggested as possible mechanisms of the bacterial immune evasion (Meri et al., 2005; Verma et al., 2006; Barbosa et al., 2009; da Silva et al., 2015; Siqueira et al., 2017). Moreover, several leptospiral proteins that could interact with C4BP, factor H, vitronectin, C7, C8 and C9 have been identified, demonstrating the presence of probable proteins in mediating the evasion of complement system at distinct levels (Verma et al., 2008; Castiblanco-Valencia et al., 2012; Souza et al., 2012; Domingos et al., 2012; Siqueira et al., 2013; da Silva et al., 2015; Siqueira et al., 2017). To assess if rLIC13259 contributes to leptospiral host's immune evasion by interacting with the components C6, C7, C8, C9 of the terminal complement pathway, as well as with the complement regulators FH, C4BP and vitronectin, the purified human components were immobilized onto microdilution plates, followed by incubation with rLIC13259 protein. The attachment was measured with anti-rLIC13259 antibodies. As visualized in Fig. 6A, rLIC13259 displayed a significant binding to C6, C7, C8, C9 and vitronectin proteins when compared with the control protein BSA. However, these interactions were only confirmed for C7, C8, C9 and vitronectin when the reaction was evaluated with the corresponding polyclonal serum

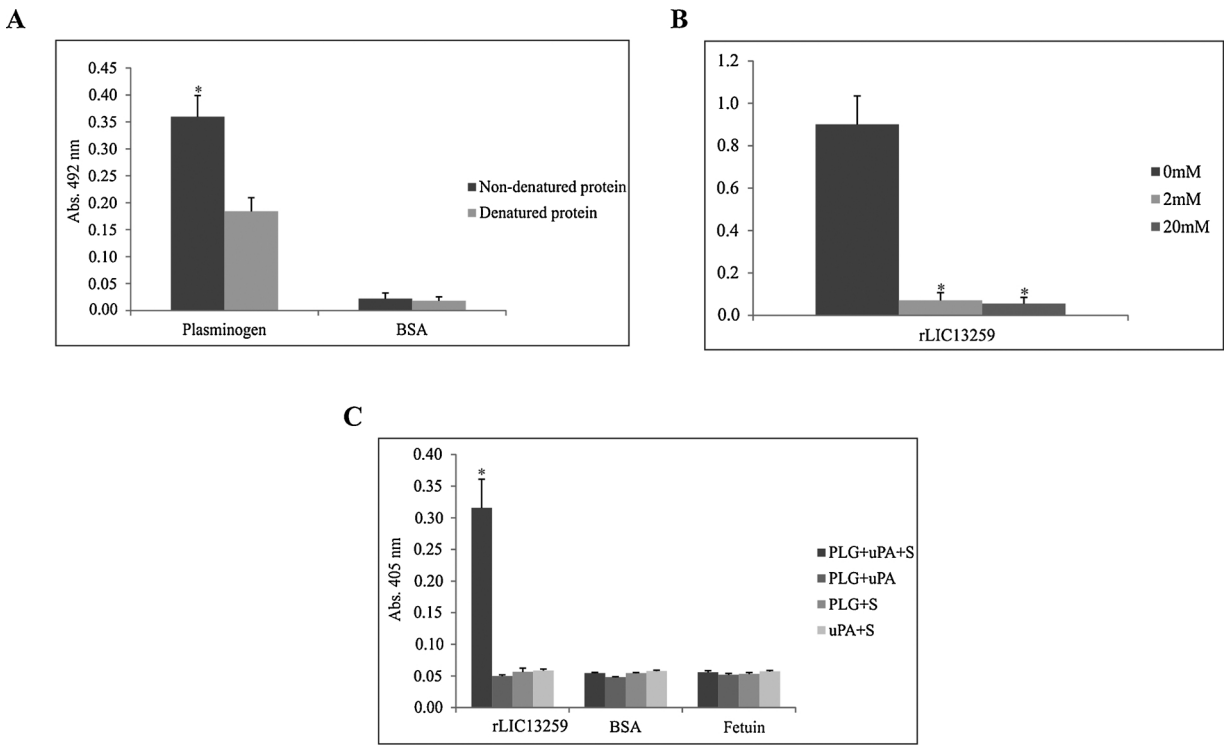

Fig. 5. Binding characterization of rLIC13259 to human plasminogen. (a) One microgram of plasminogen was immobilized on microplate, and the recombinant protein native or denatured for 10 min at 96 °C, was added. ELISA plates were then incubated for 2 h at 37 °C and bound protein was detected with polyclonal mouse anti-serum against rLIC13259. Mean absorbance values at 492 nm (± the standard deviations of two independent experiments) were compared to those obtained with untreated recombinant protein. (b) Plasminogen (1 μg/well) was immobilized into microplates and incubated with 1 μg recombinant protein in the presence (2 or 20 mM) or absence of lysine analog ε –ACA. The binding was detected using polyclonal mouse anti-serum against rLIC13259. Bars represent the mean ± SD absorbance at 492 nm of three replicates and are representative of two independent experiments. For statistical analyses, the interaction of recombinant protein with plasminogen in the presence ACA was compared with the interaction in the absence of those by two-tailed *t*-test (*p < 0.05). (c) The generation of plasmin of recombinant protein-bound plasminogen was accessed. The recombinant protein was immobilized into microplate and incubated with plasminogen (PLG), urokinase-type plasminogen activator (uPA) and specific plasmin substrate (S) to indirect measure the plasmin activity. We employed controls lacking one of the components; BSA and fetuin were also employed as negative controls. Bars represent the mean ± SD absorbance at 405 nm, as a measure of relative substrate degradation ± standard deviation of three replicates for protein, and are representative of two independent experiments.

against each component (Fig. 6B). The interaction between the rLIC13259 protein and C7, C8, C9 and vitronectin was also confirmed by Western blottings, when each component was submitted to SDS-PAGE, followed by incubation with rLIC13259 and the bindings probed with anti-rLIC13259 antibodies (Fig. 6C). To evaluate the binding of rLIC13259 with these purified proteins on a quantitative basis, an ELISA was performed allowing increasing concentrations of rLIC13259 to adhere individually to a fixed amount of C7, C8, C9 and vitronectin. A dose-dependent interaction was observed when increasing concentrations of rLIC13259 were allowed to interact with C7 (Fig. 6D), C8 (Fig. 6E), C9 (Fig. 6F) and vitronectin (Fig. 6G). Binding saturation was reached only for interaction with C8 protein, with a K_D value of 656 ± 141.4 nM. The next step was to verify if the rLIC13259 protein could capture C7, C8, C9 and vitronectin directly from NHS. Thus, the binding with increasing concentration of NHS was evaluated. Gelatin was used as negative control. A dose-dependent interaction was observed, suggesting that rLIC13259 can acquire C7, C8, C9 and vitronectin under physiological conditions (Fig. 7). These results suggest that binding of rLIC13259 to these components may be important for leptospiral survival to innate immune attack.

3.8. Binding characterization of C7, C8, C9 and vitronectin human components with rLIC13259 protein

The presence of heparin binding domains in these components has been previously described (Liang et al., 1997; Yu et al., 2005). Thus, to assess whether the binding of rLIC13259 to these components occurs via heparin sites of the components, we set out the reaction with increasing amounts of heparin prior to the binding with rLIC13259. A significant inhibition was observed when increasing amounts of heparin were added to vitronectin, suggesting that this interaction occurs via heparin-binding sites. For C7, C8 and C9 proteins, inhibition was not consistent and observed only when high concentrations of heparin were added (Fig. 8A).

3.9. rLIC13259 inhibits zinc-induced polymerization

The rLIC13259 seems to be important for leptospires by interacting with multiple proteins of the terminal complement pathway. To evaluate whether rLIC13259 could impair C9 polymerization, rLIC13259 protein was incubated with purified C9 and polymerization was induced after addition of $ZnCl_2$. As shown in Fig. 8B rLIC13259 was able to inhibit C9 polymerization in a dose-dependent manner. In the presence of 5 μg of rLIC13259, polymerization was completely abolished. BSA, used as control protein, did not affect the formation of C9 polymers. rLIC13259 protein features the ability of binding multiple host molecules, suggesting their participation in leptospiral host immune evasion process.

3.10. rLIC13259 probable mechanism to mediate leptospiral immune evasion via terminal pathway of complement system

C5-convertases generated by classical, alternative, and/or lectin pathways, cleave C5 molecules and produce C5a and C5b fragments.

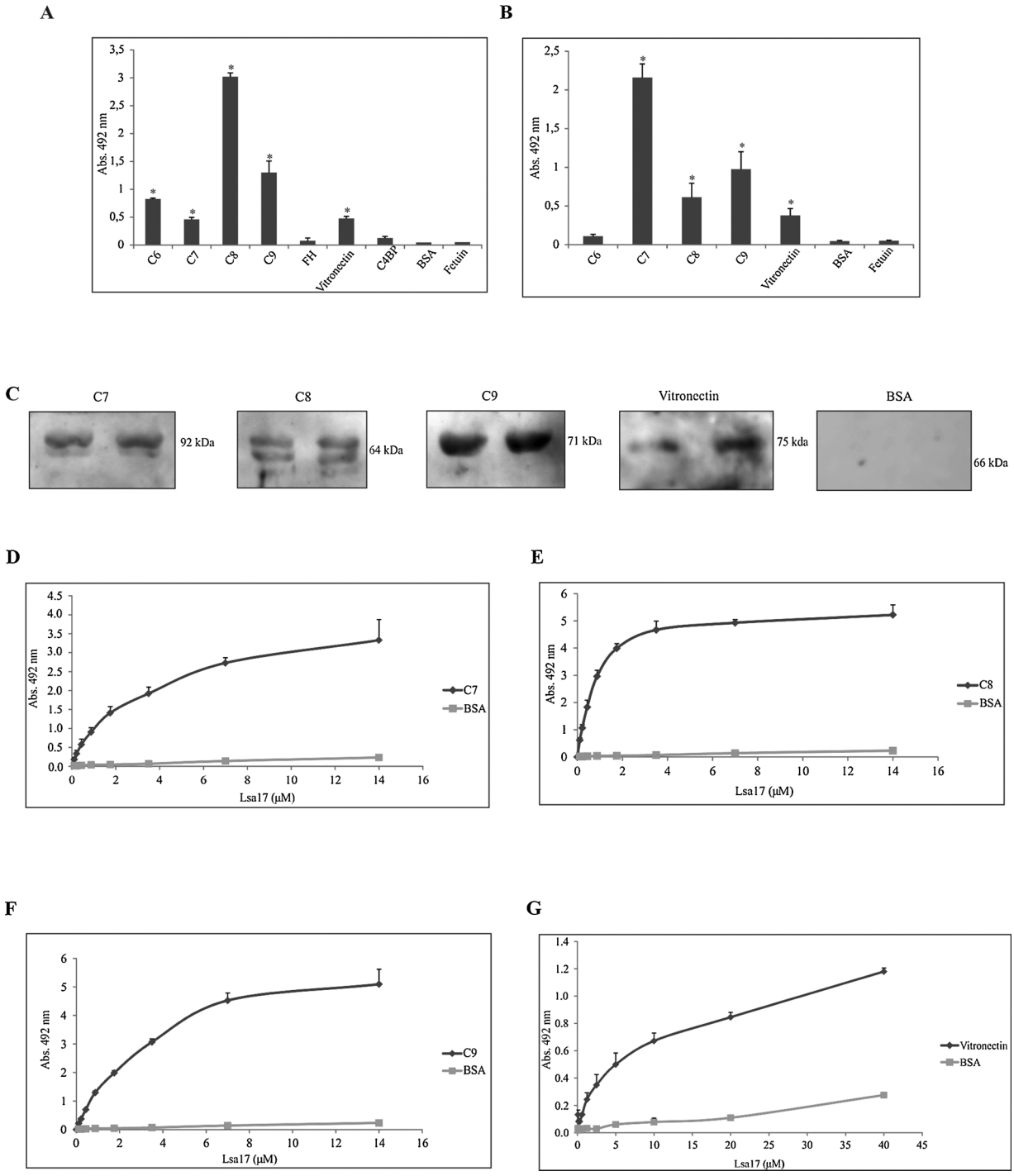

Fig. 6. Interaction of recombinant protein with human components of the terminal complement system. (a) Microplates were coated with complement system components or the control protein BSA. Recombinant protein rLIC13259 was added and component binding was detected by incubation with anti-recombinant polyclonal antibodies. (b) Microplates were coated with rLIC13259 or the control protein BSA. Complement system components were added and component binding was detected by incubation with polyclonal antibodies against each component. Bars represent the mean ± SD absorbance at 492 nm of three replicates for protein and are representative of two independent experiments. For statistical analyses, the interaction of recombinant protein with plasma components were compared to its binding to BSA (negative control) by two-tailed *t*-test (* $p < 0.05$). (c) C7, C8, C9, vitronectin and BSA were subjected to SDS-PAGE, transferred into nitrocellulose membrane, followed by incubation with rLIC13259; the bindings were probed with anti-recombinant polyclonal antibodies. Dose-dependent bindings were performed by adding increasing concentration of recombinant protein rLIC13259 to a fixed concentration of previously coated (d) C7, (e) C8, (f) C9 and (g) vitronectin. Each point represents the mean absorbance at 492 nm ± SD of three replicates and is representative of two independent experiments.

C5b initiates the terminal pathway and allows the association of C6 and C7 molecules. Component C7 is inserted into the lipid bilayer of the microorganism membrane. The interaction of C8 leads to stability of the C5b-7 complex. The association of several C9 molecules produces MAC, generating C5b-9 complex (Fig. 9). The MAC forms a pore in the plasma membrane, making possible the exit of ions and water, leading to cellular rupture and death of microorganism (Müller-Eberhard, 1986; Walport, 2001). As rLIC13259 protein was capable of binding to C7, C8 and C9 molecules, it could avoid the formation of C5b-9 complex and probably blocking the pore formation (Fig. 9). Furthermore, as

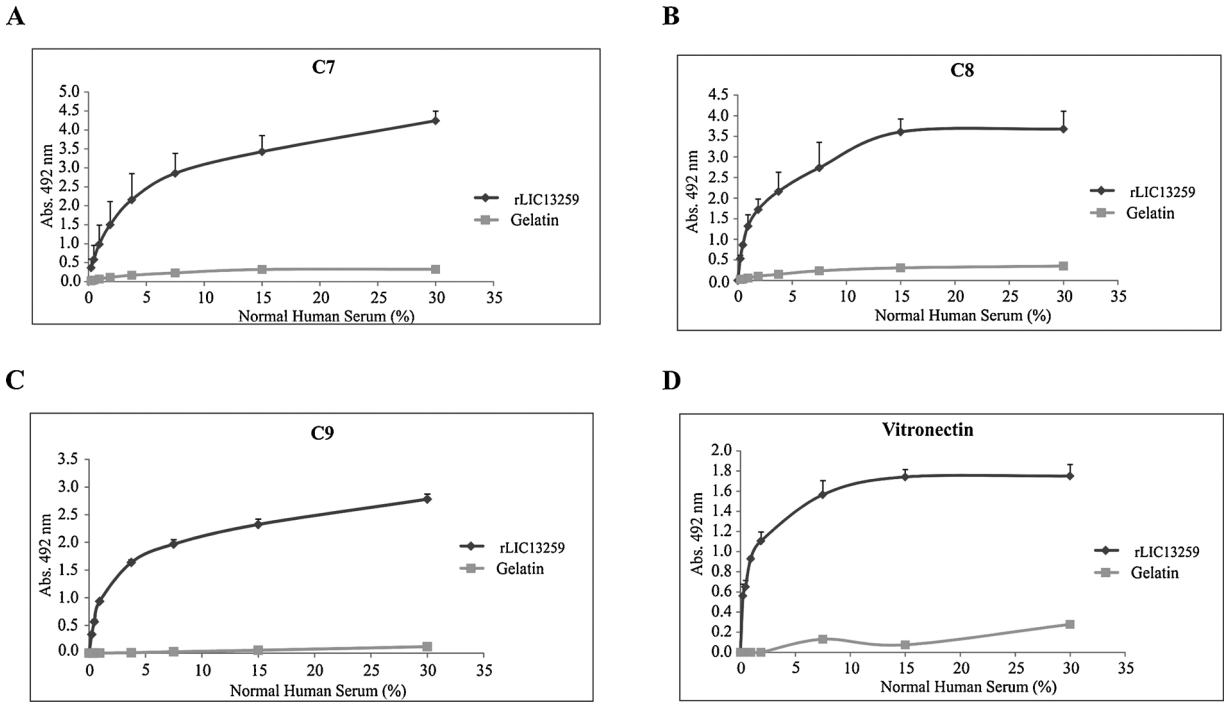

Fig. 7. rLIC13259 recruits components of the terminal complement system from NHS. One microgram of recombinant protein was coated onto microtiter plates and incubated with different amounts of NHS (0–30%). Gelatin was used as negative control for non-specific binding. Binding was detected with antiserum against (a) C7, (b) C8, (c) C9 and (d) vitronectin. Each point represents the mean absorbance at 492 nm ± SD of three replicates and is representative of two independent experiments.

rLIC13259 bound to vitronectin, the terminal pathway regulator, it is possible that this association also contributes to the inhibition of MAC formation.

4. Discussion

The successful establishment of an infection by pathogenic microorganisms can be attributed to the ability of these microorganisms to adhere, colonize, invade and, most importantly, survive the host immune attack (Blom et al., 2009; Lambris et al., 2008; Rooijakkers et al., 2007; Zipfel et al., 2007). The pathogenic *Leptospira* have a broad repertoire of proteins with the potential to participate in adhesion, invasion and complement immune system evasion. Some of these proteins have multiple roles, capable of binding to ECM, plasma components and complement system proteins, contributing to diverse levels of the leptospiral pathogenesis process (Choy et al., 2007; Castiblanco-Valencia et al., 2012; Vieira et al., 2010b; Souza et al., 2012; Teixeira et al., 2015; Domingos et al., 2012; Silva et al., 2016; Siqueira et al., 2016, Figueredo et al. 2017, Pereira et al., 2017; Santos et al., 2018). In this study, we demonstrate that rLIC13259 is a novel multifunctional protein capable of binding plasminogen and components of the terminal human complement pathway, suggesting its potential to contributing to invasion and immune evasion processes within the hosts. Most importantly, a higher LIC13259 gene expression was detected in virulent *L. interrogans* strain Fiocruz L1-130 when compared to the culture-attenuated strain M20, suggesting a possible role in virulence.

Several proteins have been identified as laminin-binding, and their biological roles are under study in our laboratory, using the saprophyte *L. biflexa,* as surrogate. Some of these proteins also exhibit collagen, fibronectin and elastin binding properties and can certainly contribute at the initial steps of host-leptospires interactions (Hauk et al., 2008; Choy et al., 2007; Lin et al., 2011; Oliveira et al., 2011; Pinne et al., 2012; Oliveira et al., 2010; Atzingen et al., 2008; Vieira et al., 2010b; Souza et al., 2012; Fernandes et al., 2012; Pinne et al., 2010). Unlike the previously reported putative adhesins, the coding sequence LIC13259 is most probably not involved in leptospiral adhesion process, since the interaction laminin and rLIC13259 was weak.

The interaction between pathogens and the host's fibrinolytic system has been suggested to be an important virulence process that facilitates the initial dissemination within the hosts (Lähteenmäki et al., 2001). Pathogens can capture PLG on their surface, followed by activation to PLA, increasing their proteolytic power, a characteristic that has been shown to contribute to ECM degradation, tissue penetration and invasion. Our group has reported, for the first time, that Leptospira species are capable of binding PLG and generating PLA, in the presence of PLG- activator (Vieira et al., 2009). The rLIC13259 has the capacity of mediating this binding, and PLG-bound to the recombinant protein could be converted into plasmin in the presence of PLG-activator. Moreover, this protein is efficient in acquiring this component in a more physiological condition, from NHS. The interaction of rLIC13259 with PLG appears to occur through the lysine-binding sites of PLG kringle domains. Several leptospiral proteins have been shown to act as PLG-binding proteins at the bacterial surface, and these interactions probably occur via PLG kringle domains (Vieira et al., 2010a; Domingos et al., 2012; Teixeira et al., 2015; Fernandes et al., 2015).

The complement system is the first line of defense of the host immune response. It comprises more than 30 distinct plasma proteins and cell bound proteins that form three major pathways: the classical, alternative and lectin pathway. Activation of any pathway results in the formation of the cytolytic MAC. Terminal complement pathway starts after C5 cleavage by C5 convertase, generating C5a and C5b. The last fragment forms a complex with soluble C6 and begins the assembly of transmembrane pores after associations between C5b-6 and C7, C8 and C9 (Preissner et al., 1985). C8 form a complex of two proteins, C8β responsible for mediates incorporation of C8 to the membrane-associated C5b-7 complex, allowing thus C8α-γ to be inserted into the lipid bilayer. Following, C8α-γ induces the polymerization of 10–16 molecules of C9, generating MAC, leading to cell lysis (Stanley, 1989; Müller-Eberhard, 1988). Excessive complement activation on self-tissue has severe effects, thus to protect the host, complement activation is

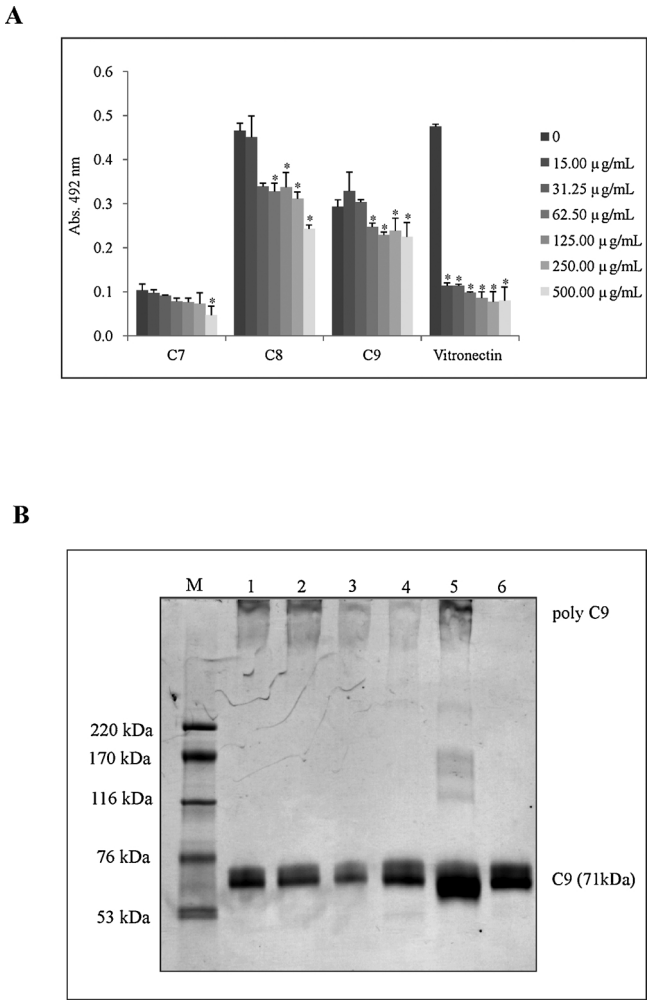

Fig. 8. Binding characterization of human C7, C8, C9 and vitronectin with rLIC13259 protein and inhibition of zinc-induced C9 polymerization by rLIC13259. (A) The effect of heparin (0–500 µg/ml) on binding of C7, C8, C9 and vitronectin to immobilized rLIC13259 was assayed. Each bar represents the mean absorbance value at 492 nm the SD for 3 independent experiments. For statistical analyses, the interaction of recombinant protein with each component in the presence of heparin was compared with the interaction in its absence by two-tailed *t*-test (*$p < 0.05$). In (B) C9 was incubated with rLIC13259 (1.25–5 µg) (lanes 2–4) or with negative control protein BSA (2.5 µg) (lane 5) at 37 °C for 40 min before the addition of 50 M $ZnCl_2$ for 2 h at 37 °C. As a positive polymerization control, we used C9 plus zinc (lane 1), while for the negative control, purified C9 alone was employed (lane 6). Samples were subjected onto a 4–20% gradient SDS-PAGE, and bands were visualized by comassie blue staining.

tightly controlled by soluble and cell surface regulators (Kim and Song, 2006).

Vitronectin is a multifunctional glycoprotein present in the human plasma that plays important roles in the complement system regulation, since vitronectin interacts with C5b-9 complement complex (Singh et al., 2010; Preissner and Seiffert, 1998). However, several Gram-negative bacterial pathogens, including leptospires, have developed the ability to interact with vitronectin, preventing MAC deposition at their surface (Singh et al., 2010; da Silva et al., 2015; Hallström et al., 2010, 2006; Leroy-Dudal et al., 2004).

Therefore, the complement system is a key target for evasion strategies developed by invading pathogens. Johnson and Muschel (Johnson and Muschel, 1965) showed for the first time that saprophytic strains were susceptible to bactericidal activity of NHS, while pathogenic species were resistant. The complement system on NHS was responsible for the bactericidal activity observed with non-pathogenic leptospiral strains, since this activity was lost with heat- inactivated NHS (Meri et al., 2005). Since then, it is assumed that immune evasion is important for leptospires virulence. However, only recently it has been demonstrated that pathogenic leptospires are able to bind soluble complement- regulatory proteins FH, FHL-1, FHR-1, C4BP, vitronectin, and several terminal complement proteins (Siqueira et al., 2017). rLIC13259 protein was able to mediate the interaction with vitronectin via the heparin binding regions, present in the vitronectin molecule. These results are similar to the previous works reported with *Neisseria* surface protein Opc (Sa E Cunha et al., 2010), *Haemophilus* PE (Hallström et al., 2009), *Moraxella* UspA-2 (Attia et al., 2006), *Leptospira* LcpA (da Silva et al., 2015) and *Pseudomonas* Lpd (Hallström et al., 2015). Interestingly, rLIC13259 can acquire vitronectin from NHS in a dose-dependent and saturable fashion, fulfilling the component-ligand property.

The rLIC13259 protein also binds to the terminal complement components C7, C8 and C9, either human purified or from NHS, in a dose-dependent manner. Several proteins have been demonstrated to be a C9 binding protein, and, hence, capable of inhibiting Zn^{2+} -induced polymerization, such as *Leptospira* LcpA (da Silva et al., 2015), *Pneumococcal* PGK (Blom et al., 2014), *Trichinella* Paramyosin (Zhang et al., 2011), *Borrelia* CspA, BGA66 and BGA71 (Hallström et al., 2013, Hammerschmidt et al. 2016). rLIC13259 was also able to inhibit Zn^{2+}-induced polymerization in a dose dependent manner, hampering MAC formation.

In conclusion, our results have described rLIC13259 as a novel surface-exposed protein capable of binding to plasminogen, vitronectin and C7, C8 and C9 complement proteins. Thus, rLIC13259 could be involved in adhesion and invasion process by plasmin generation. Most importantly, the interaction of rLIC13259 with vitronectin and C9, may contribute to immune evasion of the host, by preventing MAC deposition on the bacterial surface. To the best of our knowledge, rLIC13259 is the first leptospiral protein characterized to date with the capacity to contribute at diverse stages of the infection process, acting as a multifactorial surface protein.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of date and material

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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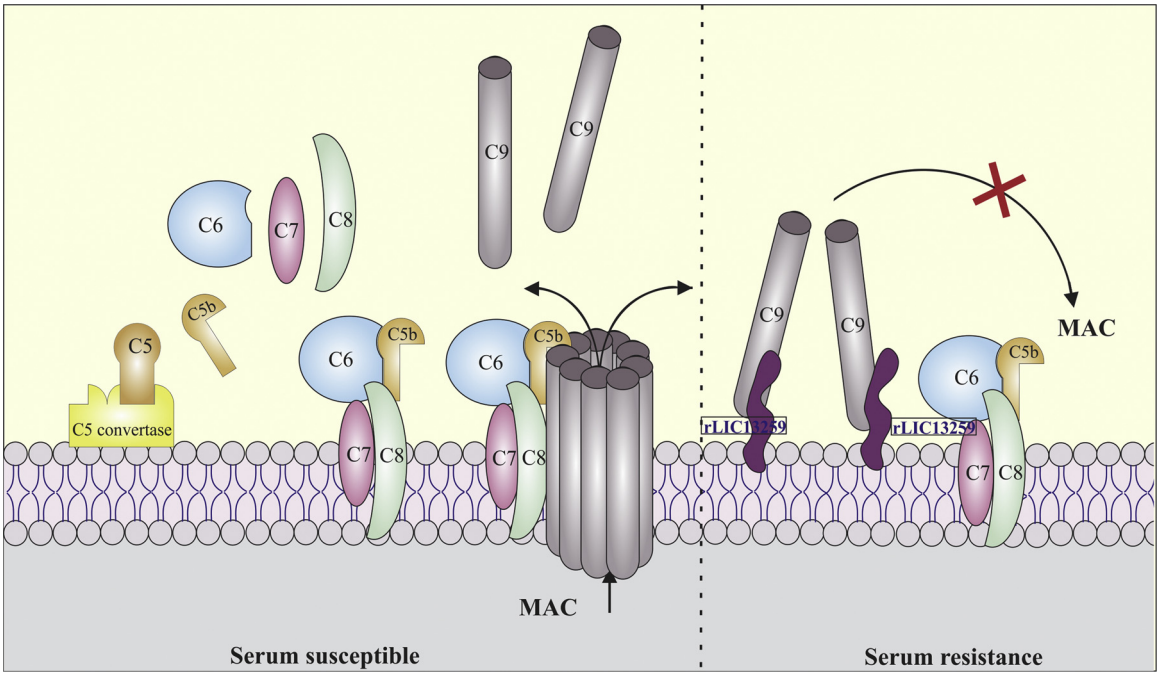

Fig. 9. Schematic representation showing the expected role of rLIC13259 in mediating leptospiral immune evasion through terminal pathway. C5-convertases generated by classical, alternative, and/or lectin pathways, cleave C5 molecules and produce C5a and C5b fragments. C5b initiates the terminal pathway and allows the association of C6 and C7 molecules. Component C7 is inserted into the lipid bilayer of the microorganism membrane. The interaction of C8 leads to stability of the C5b-7 complex. The association of several C9 molecules forms MAC, generating C5b-9 complex (Fig. 9). rLIC13259 is capable of binding to C7, C8 and C9 molecules avoiding the formation of C5b-9 complex (see Fig. 8B).

Authors' contributions

All authors participated in the literature revision, discussion and preparation of manuscript, including figures.

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